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Cancer

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13. ABSTRACT (Maximum 200 words) The project has 2 interconnecting aims: (i) to confirm and extend the observations, that apoptosis is increased and proliferation is decreased in primary breast cancer shortly after chemo and endocrine therapy, such that the predictive power of these changes for clinical response can be assessed, (ii) to develop an automated method for analysing apoptosis in fine needle aspirates (FNAs) taken from breast carcinomas. Aim (i): we have confirmed that apoptosis significantly increases 24 hours after starting chemotherapy and demonstrated that proliferation also falls by a mean of approximately 30% at this time. Change in apoptosis after 24h was not closely related to clinical response in a trial in which patients also received tamoxifen. A trial in which tamoxifen is excluded has been initiated. Patients who are c-erbB2 positive have a reduced likelihood of showing an increase in apoptosis after 24 hours. Aim (ii) flow cytometry is unlikely reliably to separate the apoptotic population of cells from normal cells but our preliminary work with Laser Scanning Cytometry indicates that this approach should be applicable to FNAs. Apoptotic MCF7 cells after camptothecin treatment can be quantified by LSC in a manner which allows morphologic confirmation.					
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FOREWORD

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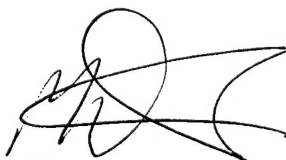
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INTRODUCTION

Preoperative or so-called neoadjuvant medical therapy provides a unique setting for the "bioclinical" study of breast cancer. Biological determinants of response to therapy may be studied in the tumour and related to clinical response in the same lesion. These studies may be made by radiological approaches such as magnetic resonance spectroscopy or positron emission tomography but most data, including ours, have been derived from immunocytochemical pathological studies of small tissue biopsies in core cuts or fine needle aspirates.

The grant proposal for this work (in late 1996) was made shortly after our finding that significant increases in apoptosis occurred within 24 hours of starting chemotherapy (Ellis et al, 1997). We had also conducted reproducibility studies using 14-gauge core-cut biopsies which indicated that at least 50% changes in apoptosis or proliferation (as measured by Ki67 staining) were required for changes in an individual tumour to be significant with 95% confidence (Ellis et al, 1998). In this original study a trend to a downward change in proliferation after 24 hours chemotherapy was not statistically significant. We also knew that significantly reduced proliferation in patients on hormonal therapy occurred in the first few weeks of treatment (DeFriend et al, 1994; Clarke et al, 1993) but the relationship with clinical response was unclear. We therefore wished to address the possibility that these early changes in the key determinants of tumour growth, which predate measurable clinical response, could be used to predict response and optimise patient therapy.

In the first year of our programme of work we confirmed that apoptosis is increased after 24 hours in approximately 70% of patients. We also established for the first time that median proliferation (Ki67) is reduced at 24 hours from 28.5% to 16.9% ($p = 0.009$). However, the variability of this effect meant that only 9/35 patients individually had a statistically significant fall. Importantly, we were able to demonstrate that after 21 days, when changes in proliferation are greater this is strongly related to response to chemotherapy, tamoxifen therapy and chemo-endocrine therapy (Dowsett et al, 1999). Also in the first year our attempts to derive an automated method to determine apoptotic index in fine needle aspirates (FNAs) by adapting the TUNEL technique to flow cytometry had been assessed as having failed and we were about to explore the same method

using Laser Scanning Cytometry. An application to fund the purchase of a Laser Scanning Cytometer (LSC) was made to the US Army Medical Research Acquisition Activity but this was not funded due to budget constraints. We have there continued to use the instrument of the Imperial Cancer Research Fund. We expect to purchase an instrument using local funds in the first quarter of 2000.

In the second year we have

- (1) assessed the potential for the LSC to be utilised for the analysis of apoptosis and extended this assessment to the quantitation of protein expression, which may become a preferred end-point instead of apoptosis itself.
- (2) completed a study of changes in proliferation and apoptosis after 24h. in 50 patients on neoadjuvant therapy and related this to tumour response.
- (3) continued to collect FNAs for analysis by the optimised technique from patients at intervals after starting medical therapy in studies of chemotherapy, chemoendocrine therapy and a randomised study of different endocrine therapies.
- (4) begun to assess changes in the expression of proteins associated with the control of apoptosis immediately after chemotherapy.

BODY:

Experimental methods:

Core-cut (14 gauge) biopsies and fine needle aspirates (FNAs) have been derived from consenting patients with primary breast cancer during their participation in one of the following clinical trials of 3 months preoperative medical therapy (only those relevant to year 2 of the grant award are mentioned).

- (i) A total of 50 patients receiving neoadjuvant adriamycin + cyclophosphamide (AC) or epirubicin + cisplatin + 5-fluorouracil (ECF). All of these patients received tamoxifen after the 24 hour biopsy.
- (ii) Patients randomised to tamoxifen, arimidex or a combination of the two. Biopsies from the first 89 patients have been derived; the study will complete at 150 patients.
- (iii) a randomised study of navelbine + epirubicin versus AC versus navelbine + mitoxantrone. This has recently incorporated a second randomisation for ER positive patients to tamoxifen or no tamoxifen.

Core-cut biopsies were fixed in formalin and embedded in paraffin-wax. Histological sections (3 μ m) were taken onto charged slides. FNAs were suspended in tissue culture medium (minimum essential medium + 25 mM HEPES) and portions were taken for cytopsin preparation and flow cytometry. Cytopins were stored at -70°C. Aliquots for LSC were fixed in 1% paraformaldehyde/PBS and then in 70% ethanol and stored at -20°C. Sections were stained using the MIB-1 antibody to the Ki67 nuclear proliferation antigen and by the TUNEL technique for apoptosis (Ellis et al, 1998).

The cytopsin preparations were stained with the MIB-1 antibody (Makris et al, 1998). The aliquots for flow cytometry were subjected to propidium iodide staining and TUNEL assay as described in detail elsewhere (Dowsett et al, 1998).

Ki67 and apoptotic index (AI) were expressed as % ages of positively staining cells. For Ki67 at least 1,000 cells were counted and for AI at least 3,000, when sections were used. FNA estimates of Ki67 required at least 100 cells.

MCF7 cells were grown for preliminary studies with LSC in Dulbecco's minimal essential medium plus 5% foetal calf serum and 10µg/ml insulin. At 50% confluence the cell cultures were exposed to dosages of ICI 182780 (pure steroidal antioestrogen) between 10^{-7} M and 5×10^{-6} M to alter protein expression and cause apoptosis. A later study of induction of apoptosis used camptothecin. Cells were then treated as cytopins and stained with antibodies to ER (DAKO 1D5), PgR (Novocastra IA6) and Ki67 (DAKO MIB1) and assessed for apoptosis using fluorescein labelled antibodies.

Results:

(i) Laser scanning cytometry:

The use of flow cytometry as the analytical tool to quantify apoptosis in an automated fashion was rejected during the first year's work and it was argued that Laser scanning cytometry was likely to be a more appropriate instrument. This quantifies the number of stained cells in a preparation and the degree of staining of each cell, as well as allowing individual cells having a particular staining pattern to be relocated and examined microscopically.

Figure 1 shows the results of using the TUNEL assay on MCF7 cells after camptothecin. The FITC integral indicates the amount of fluorescent nucleotide incorporated into the DNA and the PI integral the amount of DNA per nucleus. Each spot represents one cell. The images on the right-hand side of Figure 1 were derived from cells in the two boxes designated normal and apoptotic. The morphology of the nuclei is indicative of the validity of this designation.

There is a strong possibility that changes in proteins associated with the initiation and execution of the apoptotic process may be more reliable indices of changes in apoptosis than

the presence of apoptotic bodies themselves, because the latter are cleared very rapidly by macrophages. Thus we have been keen to extend the LSC to the quantitation of protein expression. We selected 3 proteins commonly assessed in breast cancer: ER, PgR and Ki67. Their expression was assessed and compared using conventional immunohistochemistry and LSC. Similar changes were observed with both methods (Figure 2). It is likely that the LSC more closely represents the quantitative change since it is non-subjective and fluorescence allows linear quantitation. The above work was presented at the Nottingham International Breast Cancer Symposium, September 1999 (Zabaglo et al, 1999).

(ii) Changes in apoptosis and response:

87 patients consented to enter the study of 24 hour biopsies in association with ECF/AC therapy. 53 pairs of core-cut biopsies were adequate for measurement of apoptosis. The relationship with subsequent response over the 3 months prior to chemotherapy has been assessed.

The median apoptotic index prechemotherapy was 0.8% (range 0.07 - 2.42) and after chemotherapy was 1.14% (0.18 - 4.1) ($p = 0.0001$). 32 patients (64%) had a change in apoptotic index of greater than 50%, a threshold which we have previously established as indicating a statistically significant change in apoptosis. The change in apoptosis was not significantly related to response to treatment. The 14 patients that had a complete response and a mean change in apoptosis of 162.5%. The 30 that had a partial response also had a mean change of 162.5%. The other 5 patients were categorised as non-responders and had a mean change of 84.2%. Despite the trend towards an association with response this was not statistically significant.

These results may be seen as disappointing but there was a confounding factor which may have influenced this relationship - namely the co-administration of tamoxifen with chemotherapy in almost all patients. Tamoxifen is very unlikely to have a significant impact on apoptosis after 24 hours (if at all). However, its antiproliferative effects over the three-

month treatment period are certain to have some impact on clinical response. This is likely to have the effect of dissociating any relationship between apoptotic change and response: it has been noted that best response to chemotherapy occurs in patients with high proliferation (low ER) and best response to tamoxifen in patients with high ER (low proliferation). To try to avoid this confounding factor an assessment of the relationship between apoptosis and response was made in just the ER negative patients. Again no significant relationship was apparent but there were only 19 patients in this analysis.

To address and avoid the influence of this confounding factor we have initiated a clinical trial in which patients are randomised to tamoxifen therapy as well as chemotherapy or just chemotherapy in the time prior to surgery. Biopsies prior to therapy and at 24 hours are being collected in association with the clinical trial.

This work was presented at the American Society of Clinical Oncology, May 1999 (Archer et al, 1999).

(iii) Change in apoptosis as a biological end-point:

Two studies have been carried out in which the change in apoptosis has been utilized as a biological end-point. In the first, c-erbB2 levels were assessed in the pretreatment biopsies of patients also having a 24 hour biopsy. Patients with positive c-erbB2 (n = 9, 23%) had a mean change in apoptosis of 34% in comparison with c-erbB2 negative patients (n = 30, 77%) with a mean change of 245%, $p = 0.02$. Thus this indicates that the early apoptotic response to ECF/AC therapy is less pronounced in patients with c-erbB2 positive disease. Further work to confirm this will be important since there is now the opportunity to treat these patients additionally with Herceptin. The work to date was presented in the San Antonio Breast Cancer Conference 1998.

In the second study which is on-going, 36 pairs of the 24-hour (Archer, 1998) samples have been stained (in collaboration with Dr. John Reed and Dr. Stanislaw Krajewski, La Jolla) for changes in activated-caspase 6, caspase 3, XIAP, DFF, Bax, Bcl-2, ER and PgR. This will enable us to assess whether any of these changes may provide a better index of the change in apoptosis than apoptotic index itself. Any positive results will thereafter be pursued using the LSC (see above).

Mutational p53 analysis is also being conducted on pretreatment FNAs to determine how this relates to apoptotic response, as it has been found to be a key factor in clinical response to doxorubicin (Aas et al, 1996).

(iv) Change in proliferation after 21 days endocrine therapy:

In the report of the first-year's work it was noted that we had initiated a study of 150 patients randomised in a double-blind manner to one of 3 endocrine neoadjuvant treatments: arimidex or tamoxifen or arimidex + tamoxifen. Ninety patients have been entered and sets of biopsies have been successfully taken from the large majority before treatment, after 2, 6 and 12 weeks' treatment. Our initial measurements of change in proliferation are shown in Figure 3. At this stage the randomisation code has not been broken but it is clear that substantial falls in Ki67 occur after 2 weeks and in most patients these are predictive of the level after 12 weeks. Measurements of apoptosis are currently being completed. Analysis of the biomarker studies in association with response will not occur until the study is unblinded after the last patient has completed 12 weeks therapy and had excision of their tumour.

FUTURE WORK:

Appropriately for the third year of this grant the work envisaged is completion of the on-going studies:

- (i) continued validation and application of the LSC to stored cytopins for measurement of apoptosis.
- (ii) collection and analysis of 24-hour biopsies from the patients randomised to chemotherapy \pm tamoxifen.
- (iii) completion of the studies of apoptotic protein expression in association with change in apoptosis.
- (iv) completion of the studies of apoptosis and proliferation in the endocrine trial.

CONCLUSIONS

Our data indicate that laser scanning cytometry is an appropriate instrument to use for further studies of apoptosis and protein expression in cytopin samples. Change in apoptosis after 24 hours can, however, be used as a biological end-point in its own right.

Clinical response after 3 months and apoptotic change after 24 hours are unrelated when chemotherapy is combined with tamoxifen.

Substantial changes in proliferation in response to endocrine therapy occur after 2 weeks and predict change after 12 weeks.

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Figure 1: LSC - apoptotic cells

MCF7 plus camptothecin - TUNEL assay

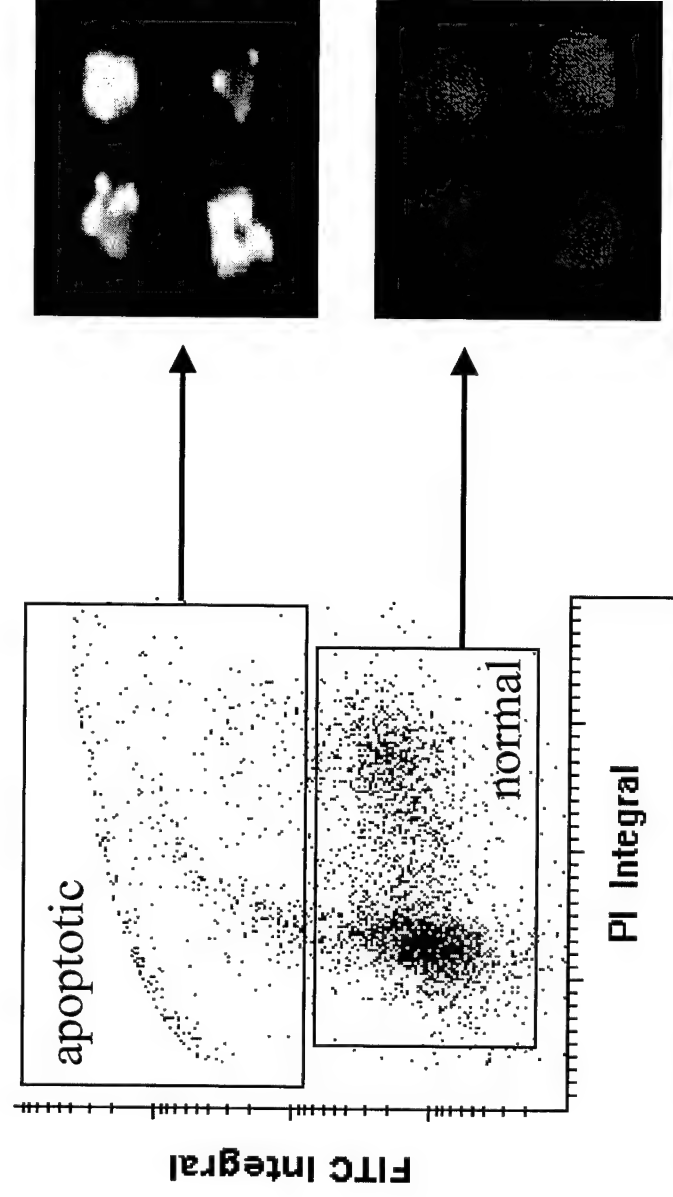
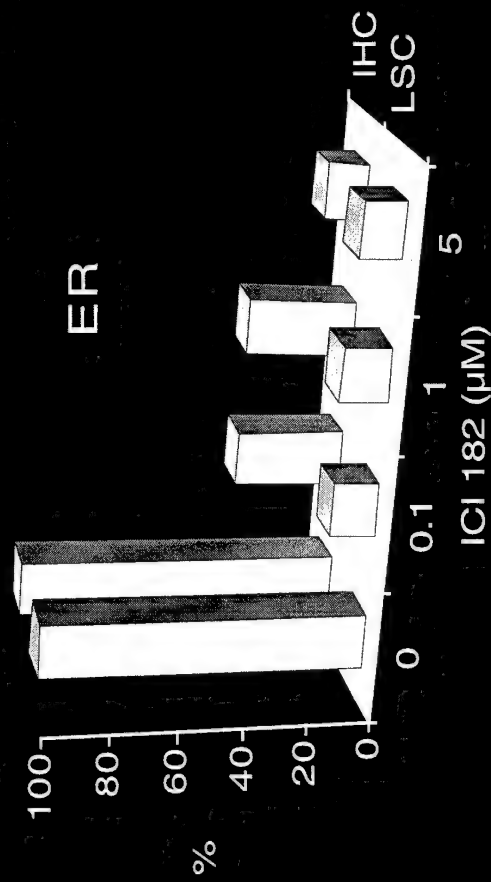


Figure 2: Comparison of LSC and IHC for assessing
ICI 182780-induced changes in MCF7 cells
Summary of results



LSC versus IHC

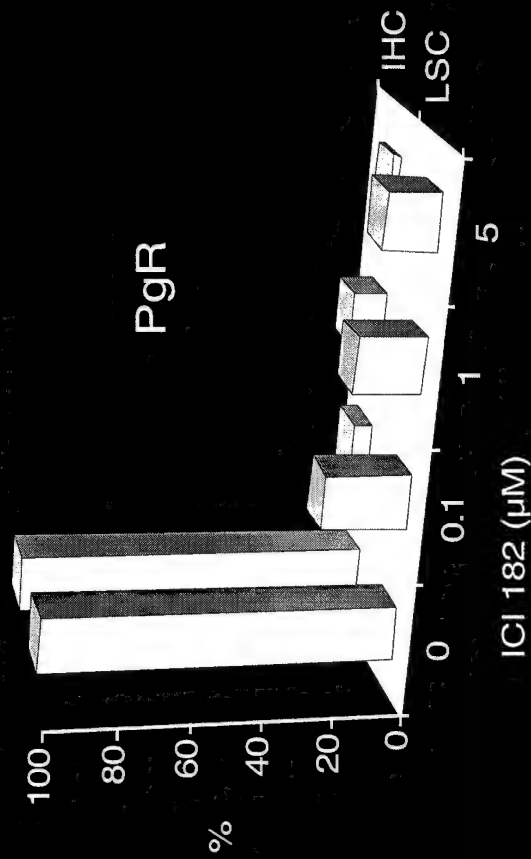
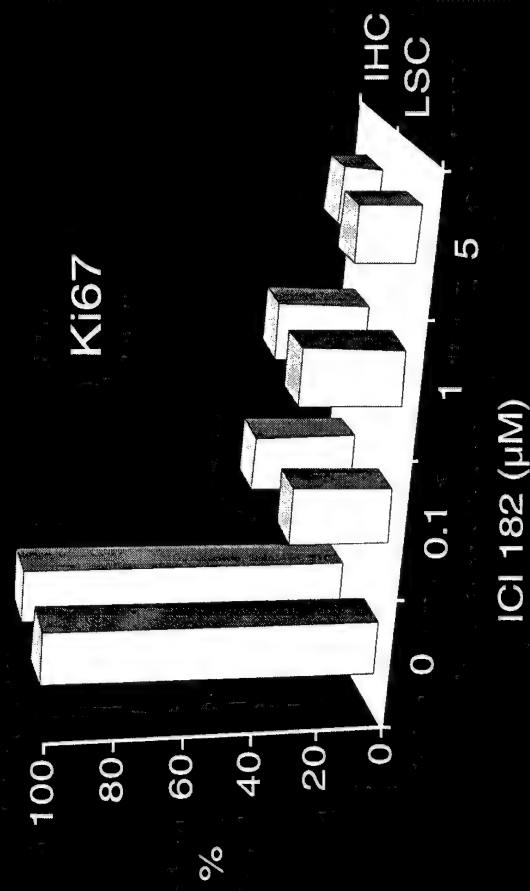
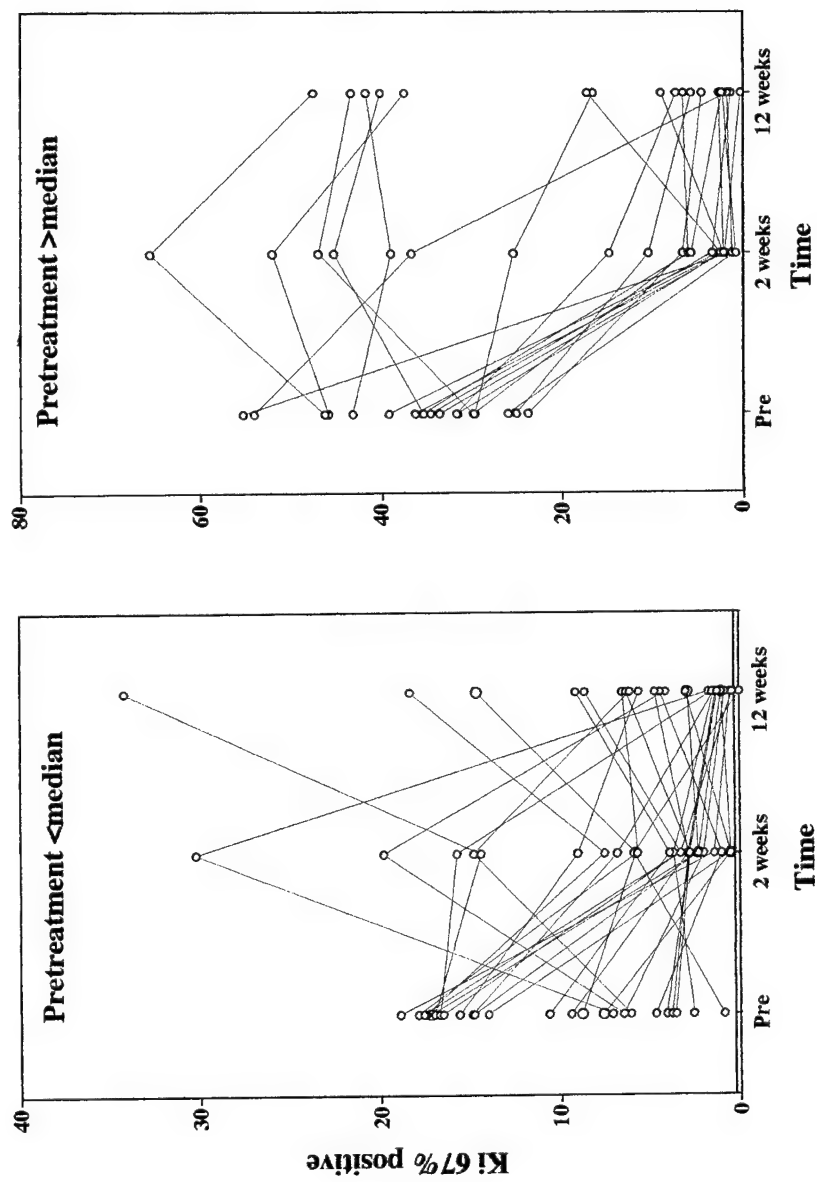


Figure 3: Change in % pos Mib over time with endocrine therapy



Appendices

This study demonstrates significantly increased angiogenesis in DCIS compared with ADH and indicates that the angiogenic process appears to be activated in breast neoplasia before invasive carcinoma is present.

096. A study of the practice of tape recording the initial oncology consultation with breast cancer patients

Brunt AM, Collis EM, Williams SA, Evans ML
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AMB started tape recording initial consultations with breast cancer patients in November 1994. It was offered when the information to be imparted was going to be particularly complex (usually incorporating trials) but is now offered to all. The impression gained was a favourable one but without formal evaluation. Therefore from 10/97 to 10/98, 136 consecutive patients who were offered a tape were prospectively assessed independently of AMB at 2 weeks and 3 months.

132 (97.1%) of 136 patients accepted the offer of the taping and all of these took it away from the consultation 84 (61.8%) of the 136 were offered entry to a trial. At 2 weeks 116 (85.3%) had listened to the tape, the number of occasions being mean 2.3, median 2 and range 0-9; whereas between 2 weeks and 3 months 59 (43.4%) listened, mean 1.3, median 0 and range 0-10. On a 4 point scale at 2 weeks/3 months the tape was assessed by 118/120 patients as 'of no use at all' 2 (1.7%)/1 (0.8%), 'not very useful' 1 (0.8%)/2 (1.7%), 'quite useful' 21 (17.8%)/22 (18.5%) and 'very useful' 94 (79.7%)/95 (79.2%) respectively. When asked if they would want the tape again with hindsight, 122/123 responded at 2 weeks/3 months YES 117/118, NO 1/1 and DON'T KNOW 4/4. Assessment of the influence of the tape in the decision to accept or refuse trial entry on a 4 point scale at 2 weeks from 71 responses was 'it had no part to play' 39 (54.9%), 'it had a little part' 13 (18.3%), 'it was a factor' 15 (21.1%) and 'strongly' 4 (5.6%). At 3 months 122 (89.7%) had kept the tape, 10 didn't and 4 could not be contacted.

Tape recording of the initial consultation with the oncologist is of benefit to most patients (and from recorded comments to carers as well). Though patients are better informed it cannot be used as a method for increasing the proportion of patients who accept the offer of trial entry.

097. Quantification of oestrogen receptors, progesterone receptors and proliferation (Ki67) by laser scanning cytometry: comparison with conventional immunohistochemistry

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Royal Marsden Hospital, London, UK

Laser scanning cytometry (LSC) is a new microscope-based technology developed to detect and quantify cell fluorescence on microscopic slides. LSC measurements of fluorescence are automatic, precise and highly sensitive and are particularly well suited to analysis of cell monolayers such as those derived from cytospin preparations of fine needle aspirates. LSC could potentially be applied to assess a variety of tumour markers. In order to validate this technology we have performed conventional microscopic scoring of immunoperoxidase-stained histological sections and of cells attached to microscopic slides, and compared these results with those obtained by LSC.

Tumour marker expression was measured on the MCF7 breast carcinoma cell line. MCF7 cells were grown in vitro under different nutrient conditions to obtain different levels of oestrogen receptors (ER), progesterone receptors (PgR) and the proliferation marker, Ki67. Fixed cells were allowed to adhere to microscopic slides and these together with paraffin

sections of cell pellets, were stained with immunofluorescence, using staining protocols developed for LSC and with immunoperoxidase.

Both methods revealed similar differences in the expression of the above markers induced by changes in nutrient conditions. Conventional immunohistochemistry (IHC) showed that PgR levels in paraffin sections of cell pellets were reduced from 58 to 14 (76% decrease, 'H' score) following oestrogen withdrawal. The subsequent addition of oestradiol (E_2) to the culture medium increased PgR levels back to 52 (271% increase). LSC analysis showed a similar pattern. Mean fluorescence (measured in arbitrary units) of fluorescein-stained PgRs decreased from 2960 to 113 (96% decrease) by oestrogen starvation, before rising again up to 798 (606% increase) after E_2 stimulation. Using IHC, E_2 stimulation caused the reduction of ER levels from 123 to 88 (29% decrease, 'H' score), whilst by LSC mean fluorescence also decreased from 11225 to 4561 (59% decrease). The percentage of Ki67 positive cells increased from 71 up to 87 (IHC) and from 60 up to 85 (LSC) following E_2 stimulation.

Although further validation studies are required, LSC may provide a faster and more objective method for scoring ERs, PgRs and Ki67 compared with conventional immunohistochemistry techniques.

098. Changes in breast tumour vascularity during primary treatment with tamoxifen

Marson LP, Kurian KK, Dixon JM, Miller WR
Breast Unit, Western General Hospital, Edinburgh, UK

Angiogenesis, or new vessel formation, is a prerequisite for tumour growth beyond 1-2 mm and for metastasis. Oestrogen may act to preserve endothelial cells by inhibiting their apoptosis and tamoxifen may exert a direct anti-angiogenic effect. The aims of the study were to determine the effects of tamoxifen on breast tumour vascularity and to assess the potential role of such changes as an early marker of sensitivity to primary endocrine therapy.

42 postmenopausal women with large (> 3 cm), operable or locally advanced ER positive breast cancers were recruited. Patients were treated with tamoxifen 20 mg daily for a period of 3 months. Core biopsies of tumour were taken before and following 2 weeks of treatment. At the end of 3 months patients underwent surgery or repeat core biopsies. Response to tamoxifen was determined by monthly ultrasound assessments for tumour volume and was defined as > 25% reduction. Microvessel counts (mvc) were performed on tumour sections following staining with antibody to factor VIII and compared at the 3 time points in association with response. 28 patients had tumours that responded to tamoxifen (67%). There was no difference in mvc between non-responding and responding tumours before, during or after treatment. There was a significant increase in mvc in non-responding tumours following 2 weeks of treatment when compared to pretreatment counts ($P = 0.03$). No significant change in mvc was observed in responding tumours.

Primary tamoxifen treatment was associated with a significant increase in tumour vascularity at 2 weeks that was not maintained for 3 months. Changes in tumour vascularity are unlikely to provide useful information as a predictor of endocrine sensitivity.

099. Changes in proliferation marker MIB1 as assessed by sequential core biopsies in primary breast cancers following neoadjuvant treatment with tamoxifen

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Changes in proliferation have been documented in breast cancer following neoadjuvant treatment with tamoxifen. However, most studies have been based upon samples taken before and after substantial periods of treatment, at which time clinical evidence of tumour response is usually evident. The

105 DIAGNOSIS OF BREAST CANCER WITH FINE NEEDLE ASPIRATION

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OBJECTIVE: Estimate the proportion of all breast cancers that might be diagnosed with fine needle aspiration (FNA) in an area with high regional use of mammography.

DESIGN: Establish the percent of women diagnosed by FNA in a case series of consecutive breast cancers according to the patients' chief complaint. Estimate the false negative rate by linkage with SEER data.

SETTING: Breast care specialty surgery practice.

PATIENTS: 219 consecutive patients diagnosed with breast cancer after referral (111 patients biopsied before referral not included).

INTERVENTIONS: None

MAIN OUTCOMES: 1.) Proportion of cancers diagnosed by FNA. 2.) Cases of cancer incorrectly diagnosed as benign by the Triple Test.

RESULTS: 86 (39%) of all cases were diagnosed by FNA; another 21 (10%) had highly suspicious FNA warranting wide excision as the initial surgery. FNA results in relation to initial complaint follow:

complaint	n	CA	susp.	Atyp.	benign	not done
routine exam	2					
pt found lump	73	46	12	3	2	10
pm found lump	14	10	1	1		2
abnl mammogram	121	25	8	6	3	79
other	9	5		1		3

SEER linkage did not identify any cancers missed by FNA Triple Test.

CONCLUSIONS: 49 percent of all breast cancers can be diagnosed by FNA without radiology image guidance, including one fourth of all lesions identified by mammography. Patients with abnormal mammograms should be considered for palpation guided FNA before image guided biopsy.

106 DETECTION OF MALIGNANT BREAST TISSUE AND LYMPHATIC NODES BY 5-AMINOLEVULINIC ACID

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Objectives: In other disciplines such as urology and dermatology superficial malignant tumours can be localized by 5-aminolevulinic acid (5-ALA) induced Protoporphyrin IX (Pp IX) fluorescence in order to increase the radicality of tumour resection. The aim of this study is to analyse the specific fluorescence behavior of breast cancer tumour and the involved lymphatic nodes.

Materials and methods: So far 11 patients (32 to 78 years) with a histologically established breast cancer have received orally 5-ALA in a dosage of 10-20 mg per kg b.w., dissolved in 100 ml H₂O, 3-5 hours before breast ablation with axillary dissection. After operation the tumours and the lymphatic nodes have been analyzed. Pp IX accumulates in high proliferating tumour tissue. Its fluorescence is excited with blue light from a filtered incoherent light source (D-Light, K. Storz, Germany) and detected through a long pass yellow filter (450nm) integrated in a cystoscope or in a modified operating microscope. After documentation, tissues of interest from the breast tumour and lymphatic nodes have been frozen and cut into 5 µm sections for H & E staining and 20µm sections for the fluorescence microscopy and compared to each other.

Results: The fluorescence effects of breast tumour and the lymphatic node metastases showed, that tumours with a high fibrotic component have a less pronounced or even negative fluorescence effect, whereas tumours with a high proliferation rate are well detectable. The lymphatic node metastases could be proofed from macroscopically relevant fluorescence effects to a 2 mm micro-metastasis.

Conclusion: Fluorescence diagnosis with 5-ALA for intraoperative detection of breast cancer tissue could help to increase the effectivity in minimal invasive tumour surgery.

107 Mammaglobin as a Novel Marker for the Detection of Human Breast Cancer.

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As the number of new cases of breast cancer increases each year, there is an increasing need to identify tumor specific markers that have an impact on predicting the clinic outcome and management of this disease. Our laboratory has identified a breast-specific gene, mammaglobin, whose expression can be identified in a large percentage of primary breast cancers. The goal of this study was to evaluate the effectiveness of mammaglobin as a specific breast cancer marker and determine the frequency and efficiency of detecting breast cancer metastatic lesions in both lymph nodes and in the circulation. Approximately 80% of primary breast cancer cells were immunopositive for mammaglobin and this observation is consistent to mammaglobin expression levels detected in primary breast cancer. The percentage of mammaglobin-positive lymph node samples from women with metastatic breast cancer exceeded 60% and the detection of mammaglobin was highly specific for breast cancer when compared to other metastatic tumor types. In an attempt to detect circulating occult breast metastases, we assayed for breast tumor cells in peripheral stem cell products derived from women with metastatic breast cancer. Using primers specific to the mammaglobin cDNA, we could detect breast tumor cells in 30% of the women with metastatic breast cancer. Peripheral stem cell products from either males or women with other cancer types were uniformly negative for mammaglobin detection. This study demonstrates that mammaglobin is an effective marker for the specific detection of human breast cancer. The sensitivity and specificity afforded by mammaglobin can be exploited to detect breast tumor occult metastases in lymph node specimens and in peripheral stem cell products. Of note, mammaglobin is a secreted protein, therefore, its potential as a serum marker for breast cancer detection is of interest.

108 C-erbB-2 POSITIVITY CORRELATES WITH POOR APOPTOTIC RESPONSE TO CHEMOTHERAPY IN PRIMARY BREAST CANCER.

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C-erbB-2(HER-2/neu) expression has been found to be associated with resistance to chemotherapy in breast cancer. Chemotherapy is thought to act via induction of apoptosis which we have demonstrated can be detected 24 hrs after starting treatment. In this study we have investigated the relationship between this 24 hr increase in apoptosis with pretreatment c-erbB-2 status in primary breast cancer. 39 patients underwent anthracycline-based combination primary chemotherapy (epirubicin or doxorubicin, 60mg/m²), consented to a core biopsy prior to, and 24 hrs after the first course and had sufficient tissue in both biopsies to be analysed. Paraffin-embedded tissue was stained using the TUNEL assay and apoptotic cells were scored as a percentage of the tumour cells. C-erbB-2 positivity was determined by the presence of immunohistochemical membrane staining of tumour cells using the monoclonal antibody ICR12. The mean apoptotic index pre-chemotherapy was 0.89% and after 24hrs was 1.79%, a mean increase of 196% (p<0.0001). Nine patients were c-erbB-2 positive (23%) and these had a significantly lower mean increase in apoptotic index at 24 hrs than the c-erbB-2 negative patients (34% vs 245%; p=0.0196). We have previously demonstrated that the threshold for a statistically significant change in apoptotic index in an individual patient is 50%. Only 3 of the 9 c-erbB-2 positive patients showed a >50% increase compared with 22 of the 30 c-erbB-2 negative patients (p=0.048).

These results indicate that c-erbB-2 positive breast cancer has a reduced apoptotic response to combination chemotherapy. This may be a mechanism of chemoresistance in this sub-group.

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Four Cycles of Adjuvant AC (Adriamycin+Cyclophosphamide) \pm 2 Cycles of EP (Etoposid+Cisplatin) in Node Positive Patients with Breast Cancer (A Randomized Trial). Fikri Içli, Hakan Akbulut, Dilek Dinçol, Handan Karaoguz, Ahmet Demirkazik, Ragıp Çam, Filiz Çay, Salim Demirci, Aytaç Uner, Mutallip Unal. Dept. of Medical Oncology, Ankara University Faculty of Medicine, Ankara, Turkey; Dept. of Surgical Oncology, Ankara University Faculty of Medicine, Ankara, Turkey and Dept. of Medical Oncology, Gazi University Faculty of Medicine, Ankara, Turkey.

Premenopausal patients with positive axillary lymph nodes following curative mastectomy were randomized to either 4 cycles of AC or 4 cycles of AC+2 cycles of EP. From May 1992 to May 1996, 165 patients were enrolled (AC:83, AC+EP:82). The doses were as follows: Cyclophosphamide 600 mg/m² iv, adriamycin 60 mg/m² iv, Etoposid 100 mg/d x 5 days iv, and cisplatin 20 mg/m² d x 5 days iv. Chemotherapy was repeated every 3 weeks. Median follow-up is 54 months. One patient from AC group was lost to follow-up. Four patients from AC+EP group and 1 from AC group were excluded because of stage IIb or IV disease. Nine additional patients from AC+EP arm refused to take EP following 4 cycles of AC. Patient characteristics including age, number of positive nodes, tumor size and estrogen receptor positivity for both groups were comparable. The results of 80 patients in the AC group and 69 in the AC+EP group are as follows:

	CA group		CA+EP group		
	No. of Patients	% relaps	No. of patients	% relaps	p
No. of lymph nodes					
1-3	34	41.2	24	9.1	0.014
4-9	27	57.7	23	60.9	1.000
≥ 10	20	80.0	22	72.7	0.723
OAS (mean, months)	61.8 \pm 2.4		61.9 \pm 3.1		0.880
DFS (median, months)	34.0 \pm 9.3		46.0 \pm 8.0		0.332

The disease-free survival (DFS) for the CA+EP group was significantly longer when compared to CA group in the subgroup of patients with 1-3 positive nodes ($p = 0.013$). No grade 4 bone-marrow toxicity was observed in both groups. Two patients in the EP group were lost due to cerebral infarct, a few days following the second cycle of EP, which may be related to cisplatin toxicity.

In conclusion, 2 cycles of EP following 4 cycles of CA seems to decrease the relapse rate in operable breast cancer patients with 1-3 positive nodes. Longer follow-up is needed to confirm this conclusion.

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High-Dose, Lenograstim (rHuG-CSF) Supported, Versus Standard Dose of Induction Chemotherapy in Non Metastatic Unilateral Breast Cancer: Preliminary Results. F. Ben Ayed, P. Chinet, J. Bonnetterre, F. Tavakoli, F. Tharaux, B. Chevalier, P. Kerbrat. Tunisia, France, Chugai-RP.

Objectives: To compare clinical and histological response, overall survival (OS) and percentage of patients (pts) able to receive scheduled chemotherapy (CT) at 21 days interval. **Method:** From 1993 to 1997, 154 pts, median age: 47.5 (23-66) with non metastatic de novo inflammatory unilateral breast cancer were randomized to receive 4 cycles of induction CT every 21 days. Group I: FEC-HD supported by lenograstim: Fluoracil 750 mg/m²/d (day 1 to 4), epirubicin 35 mg/m²/d (day 2 to 4), cyclophosphamide 400 mg/m²/d (day 2 to 4) with lenograstim 150 μ g/m²/d (day 6 to 15). Group II conventional FEC-75 alone: Fluoracil 500 mg/m² (day 1), epirubicin 75 mg/m² (day 1), cyclophosphamide 500 mg/m² (day 1). Responders to induction treatment entered loco-regional therapy (radiotherapy \pm surgery) followed by 4 cycles of FEC-75 every 28 days. Then patients were followed for at least 5 years (still ongoing). **Safety:** More non-haematological toxicities were observed in high-dose group, some of these (pain and fever) are commonly observed in G-CSF treated pts. One death due to progressive disease during FEC-75 induction. **Results:**

	Group I (n = 77)	Group II (n = 77)	p value
Pts with 4 induction cycles	85.7%	97.4%	
Nb of administered cycles at d 21	33 (15.9%)	26 (11.5%)	
Nb cycles delayed due to neutropenia	4	3	
Pts with loco-regional treatment	92.2%	92.2%	
ORR (CR + PR)	83.1%	66.2%	p = 0.02
Nb pts evaluable for histological response	60	62	
Complete Histological response pts	16.7%	6.4%	NS
Overall Survival at 36 months	66%	60%	NS

Conclusions: With lenograstim support, only few cycles were delayed due to neutropenia in FEC-HD group. A higher clinical response rate was observed after FEC-HD induction. This was not always confirmed by histology. There is no difference in 3-years OS.

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Quality Analysis of Axillary Lymph Node Dissection in Breast Cancer Patients in the Northern Part of the Netherlands. P.H.B. Willemse, R. Otter, M. Schaapveld, E.G.E. De Vries, P.L. De Vogel. Dept of Medical Oncology, University Hospital and Northern Comprehensive Cancer Center (IKN) and National Cancer Registry (NeCaRe), Groningen, the Netherlands.

Introduction. Standard axillary lymph node dissection (ALND) is increasingly being replaced by level 1 dissection or sentinel node procedures. The number of nodes with tumor involvement (pos nodes) is of value for prognosis and participation in chemotherapy protocols. The aim of the study was to analyze the quality of the ALND in the 18 community and 1 referral hospitals in the IKN region over '94-'97.

Results. 4943 patients who underwent mastectomy for breast cancer were evaluated. The total number of nodes removed (NNR > 10) increased over the 4 yrs study period from 65% to 72%. Less nodes (<10) were removed in smaller hospitals (<600 beds): NNR median 40% range 24%-61%, $n = 2514$ than in the larger ones: NNR median 21%, range 12%-31%, $n = 2429$. The number of nodes analyzed (NNA) was lower in the 4 pathology labs reporting on <200 cases/y: NNA <10 in 24%-61% vs NNA 12%-32% in the 4 larger labs; NNA >20 in 0.8%-7.2% versus NNA 1.7%-15.3% in the larger ones. The % pos nodes increased with number of nodes removed (<10: 35% pos, 10-20: 40% pos, >20: 54% pos) as well as the chance of finding >4 nodes positive (resp. 8.9%, 17.4% and 31%), irrespective of age (< or >50 y). The % positive nodes correlated with tumor size and grade.

Conclusion: Increasing the number of nodes that are removed and analyzed increased the number of pos nodes. The chance to find pos nodes depends on the hospital size and number of cases per pathology lab. About 20% of patients were understaged in smaller hospitals.

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Induction of Apoptosis and Reduction in Proliferation 24 Hours After Primary Chemotherapy in Early Breast Cancer. Caroline D. Archer, Paul A. Ellis, Janine Salter, Margaret Hills, Mitchell Dowsett, Ian E. Smith. Dept of Medicine, Royal Marsden Hospital, London; Guys, Kings, St. Thomas' Cancer Centre, London, United Kingdom and Dept. of Academic Biochemistry, Royal Marsden Hospital, London, United Kingdom.

Primary/neoadjuvant treatment in early breast cancer provides a model to study the effects of chemotherapy on the biological determinants of tumour growth. We have previously reported chemotherapy induced apoptosis on 17 patients with primary breast cancer (Ellis et al, Proc ASCO 15, 1996). This represented the first *in vivo* demonstration of chemotherapy induced induction of apoptosis in solid tumours. We now present our final data from that study. 80 patients in total consented to a core biopsy pre and 24 hours after the first cycle of primary chemotherapy. The chemotherapy used was anthracycline based; principally adriamycin and cyclophosphamide (AC) or epirubicin, cisplatin and infusional 5FU (ECF) as part of a randomised clinical trial. Of these 80 patients, 50 had enough tissue in both biopsies to be analysed. Immunohistochemical techniques were used on the paraffin-embedded material to determine apoptosis using the ISEL/TUNEL technique, and proliferation with MIB1 antibody. The indices were expressed as a percentage of the total tumour cells. The median apoptotic index (AI) pre-treatment was 0.8% (range 0.07-2.42) and at 24 hours, 1.14% (range 0.18-4.1), $p = 0.0001$. 32 patients (64%) had a change in apoptotic index of >50%. The median MIB1 score pre-treatment was 30.5% (range 0.9-93%) and post chemotherapy was 19.6 (range 0.57-90%), $p = 0.0036$. There was no significant correlation between change in apoptosis and proliferation in individual patients. The data presented here confirm our earlier finding, that apoptosis can be demonstrated to be induced 24 hours after chemotherapy in breast cancer, and also that there is a reduction of proliferation seen early after treatment. Both of these effects probably contribute to reduced tumour growth and may serve as intermediate indices of treatment benefit.

Biological Studies in Primary Medical Therapy of Breast Cancer: The Royal Marsden Hospital Experience

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Introduction

The unique opportunity for studying the biology of breast cancer during therapy which is afforded by the scenario of primary medical therapy (PMT) has been recognised for many years [1]. While studies of prognosis in relation to biological and pathological characteristics of breast tumours are widespread, they are in many cases of dubious value: there is general acknowledgement that the discovery of predictive factors, i.e. markers which are indicative of response to or benefit from a specific therapeutic manoeuvre, would be of much greater value. PMT is well suited to address this issue, since biological measurements can be made in the same tumour in which clinical response occurs, and this response is more readily measured in the primary lesion than in metastatic sites. Many "bioclinical" studies have been conducted to identify predictive markers in adjuvant therapy or in advanced disease. However, in adjuvant therapy, since patients are clinically disease-free post-surgery, it is not possible to measure the response of an individual patient. In advanced disease biomarkers are generally measured in the primary lesion and response in metastases, such that the response is temporally, topographically and possibly biologically separated from the biomarker measurements.

The majority of studies on PMT have assessed only pre-treatment markers but sequential measurements in multiple samples during therapy may be at least as valuable. The changes noted may be predictive of response and in some cases, e.g. with changes in proliferation and apoptosis, the measurements may be intimately related to growth changes, such that these might be valid intermediate indices of response.

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Additionally, although primary medical therapy is generally associated with impressive response rates, pathological complete responses are infrequent. Studies of the residual tissue after the completion of medical therapy or at the time of maximal response should provide data on mechanisms of response and resistance. A straightforward view might be that studies of such residual cell populations should be our highest priority, since it is these populations which are the threat to the patients' welfare.

At the Royal Marsden Hospital we have conducted several clinical trials during the last decade (Table 1) and a programme of biological research as an integral component of them. During this time we have performed a series of validation studies which have helped to assess the variability of analyses, and thus the degree of change needed to be seen in an individual patient for it to be deemed significant. Our most recent data have focused on changes in proliferation and apoptosis; the validation studies relevant to these are discussed below.

Sampling techniques, advantages and disadvantages

We have employed core-cut biopsies and fine needle aspirates (FNAs) as sampling techniques for obtaining pre-treatment and on-treatment tumour tissue.

FNAs can, if conducted with care, be taken at frequent intervals (every few

Table 1. Clinical trials of primary medical therapy (PMT) providing biological material for analysis at the Royal Marsden Hospital. Analyses have also been conducted on patients given PMT outside of the trial setting.

Trial design	Drugs	Study period	No. patients
Randomised PMT vs Surgery	Mitoxantrone, methotrexate, tamoxifen	1989/1995	300
	Tamoxifen	1994/1996	40
Non-randomised PMT	Cyclophosphamide, methotrexate, 5-fluorouracil	1985/1990	64
	Epirubicin, cisplatin, 5-fluorouracil (ECF)	1990/1992	50
	ECF (+ tamoxifen) vs adriamycin, cyclophosphamide, tamoxifen	1993/1998	180

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days) without undue distress to the patient. The number of cells obtained can vary substantially but may be $>10^6$. The cell suspension is particularly well suited to automated analytical techniques such as flow cytometry, but most of our analyses have been conducted on cytopins which, in the best cases, can yield several thousand cells/slide. Our early experience with FNAs demonstrated that multiple markers could be measured on a single FNA with generally good concordance with the same markers assessed by histology [2,3]. For example, 91.5% concordance was seen for oestrogen receptor (ER) when evaluated as ER⁺ or ER⁻. It was also shown that statistically significant correlations existed between ER and either PgR, bcl-2 or p53 ($r = 0.61, 0.30$, and -0.39 , respectively), between p53 and S-phase fraction (SPF) or Ki67 ($r = 0.33$ and 0.40 , respectively) and between Ki67 and S-phase ($r = 0.56$) [4]. This significant correlation between the two proliferation indices has not been reported by all groups, but was confirmed by our observation of a strong correlation between SPF in FNAs and Ki67 (measured as Mib1) in sections ($\rho = 0.59, n = 75, p < 0.0001$) [5]. It was notable, however, that SPF was not measurable in 31/164 (20%) aspirates either because of inadequate cellularity or overlapping peaks on DNA histogram [2].

A disadvantage of FNAs is that they do not allow invasive disease to be distinguished from *in situ* lesions on morphological grounds. However, in the near future advances in molecular pathology may allow analysis of a panel of markers to discriminate between the two.

Measurements of apoptosis have become a key element of our studies of response and resistance to PMT. However, the low number of apoptotic cells, even in treated tumours, makes the measurements laborious: normally ≥ 3000 cells need to be scored for sufficient statistical confidence. We have therefore attempted to automate our analyses of apoptosis by performing the TUNEL assay on cells taken by FNA, using flow cytometry for analysis [6]. In a pilot study of 12 patients we observed a significant correlation ($p = 0.03$) between the automated methodology and our standard methodology on sections. Further experience, however, has revealed two substantial difficulties with this technique: (i) the cutoff taken to define apoptotic and non-apoptotic cells without visual confirmation is unreliable; (ii) the TUNEL assay also stains fragmented DNA in areas of necrosis; this would usually be avoided in scoring apoptosis on sections but cannot be discriminated by flow cytometric approaches.

Core-cuts have the advantage of retaining the epithelial-stromal architecture of the tumour. In most of our studies a 14-gauge needle is used to provide a core which is c. 1.5 cm long by c. 2 mm diameter and weighs 20-30 mg. Each histological section through a highly cellular core can reveal $>25,000$ cells. Large numbers of sections from the same core can be available. The local trauma which may be caused, however, does not allow core-cuts to be made at frequent intervals.

Variability of analysis

The focus of our work on changes in proliferation and apoptosis has led us to evaluate the precision of the measurement of proliferation and apoptosis. Figure 1(a) and (b) show the reproducibility of measurements of Ki67 (Mib1) and apoptotic index (AI), respectively, as found in pairs of core-cuts taken immediately after one another from the same tumour. For Ki67 and AI the mean difference was 33% and 38%, respectively, and the standard deviation of the difference was 16% and 22%, respectively. In essence, for a pair of results to be considered significantly different from one another there needs to be a >50% difference between them [7].

A slightly different approach was taken to estimate the reproducibility of S-phase measurements; in this case the samples were taken 14 days apart but with no intervening treatment. The values of 25 pairs were strongly correlated, with no systematic difference between the first and second samples. This observation indicates that there are no significant alterations to SPF as a result of taking an earlier aspirate [3]. Similar data have also been derived indicating no significant change in ER, PgR, Ki67, p53 and bcl-2 in FNAs [8].

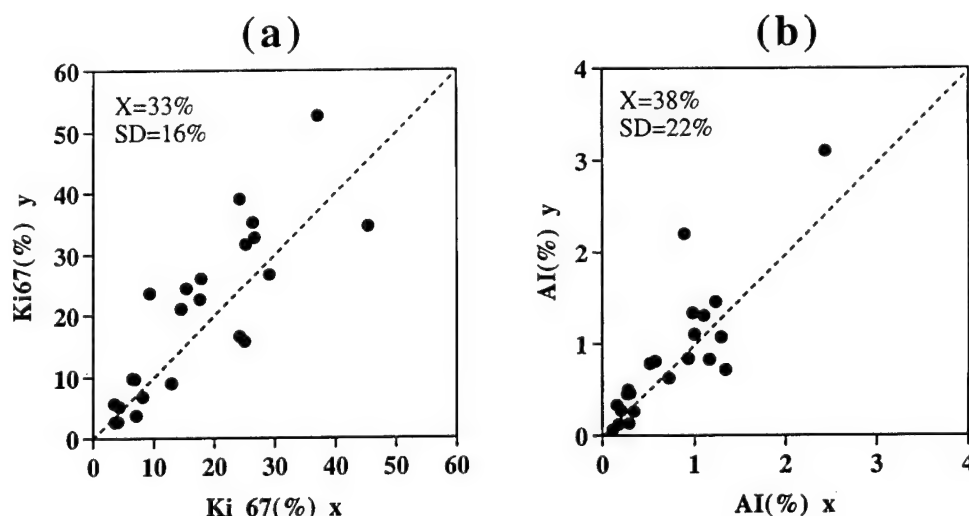


Fig. 1. Intratumoral variability of Ki67 (a) and apoptotic index (AI) (b) as shown by pairs of 14-gauge core-cuts taken immediately after one another from different sites (x and y) of the same breast carcinoma. X = mean difference, SD = standard deviation of the difference. The dashed line is the line of perfect correlation.

Endocrine therapy

Numerous studies of hormonal therapy and breast cancer have established that very few patients with ER-negative disease respond to therapy. These and other data are documented in detail in other chapters of this volume (Miller, Howell, Robertson). We have conducted a small number of studies investigating the changes which occur in what may be considered pivotal biomarkers during tamoxifen therapy.

In a series of 19 patients, nine of whom responded to therapy, ER levels in repeated core-cuts fell from $33.0 \pm 17.9\%$ cells staining to $20.5 \pm 12.7\%$ and $12.0 \pm 16.0\%$ after 1-3 and 4-6 months, respectively [9]. Progesterone receptor (PgR) levels also fell with treatment. Whilst this progressive fall in ER levels with therapy would be consistent with the selection of an ER-negative phenotype, other studies by our group indicate that development of this phenotype is not a significant feature of acquired tamoxifen resistance [10]. These data are similar to those of Robertson et al. [see chapter in this volume].

In the same study all five responding patients in whom paired Ki67 measurements were available showed a decrease in staining (mean pre-treatment: $28.0 \pm 8.4\%$; after 1-3 months, to $9.2 \pm 4.8\%$). There was no consistent change in non-responders (pre-treatment, $32.8 \pm 15.8\%$; after 1-3 months $20.0 \pm 10.4\%$; after 4-6 months $29.2 \pm 8.7\%$).

More recently we have studied changes in hormone receptors and proliferation markers in FNAs taken before and after 14 days' tamoxifen treatment. Importantly, these data confirmed the association of response with decreased Ki67 recorded above: the group of eight patients who eventually responded to treatment showed a significant reduction in Ki67 and there was no significant change in the six non-responders ($p = 0.005$) [8]. The data have been increased to a total of 22 patients (11 responders, 11 non-responders) and the relationship is maintained (Fig. 2).

These data provide support for early changes in proliferation during hormone therapy being valid intermediate markers of response. This is important given the increasing usage of this parameter in the development and comparison of new drugs [e.g. 11].

Chemotherapy

Our studies of repeat sampling of patients during their treatment with ECF (epirubicin, cisplatin and 5-fluorouracil) indicated for the first time that chemotherapy induces measurable increases in apoptosis in human breast carcinomas within 24 hours of starting therapy. Our initial report showed an overall increase in the percentage of apoptotic cells from a median of 0.47% to 1.02% ($p = 0.009$) [12]. Ten of the 17 patients showed an increase of $>50\%$, which can be considered significant for individual patients (see above). We have now confirmed that changes of this degree occur in a further series of 22 patients [13]. In

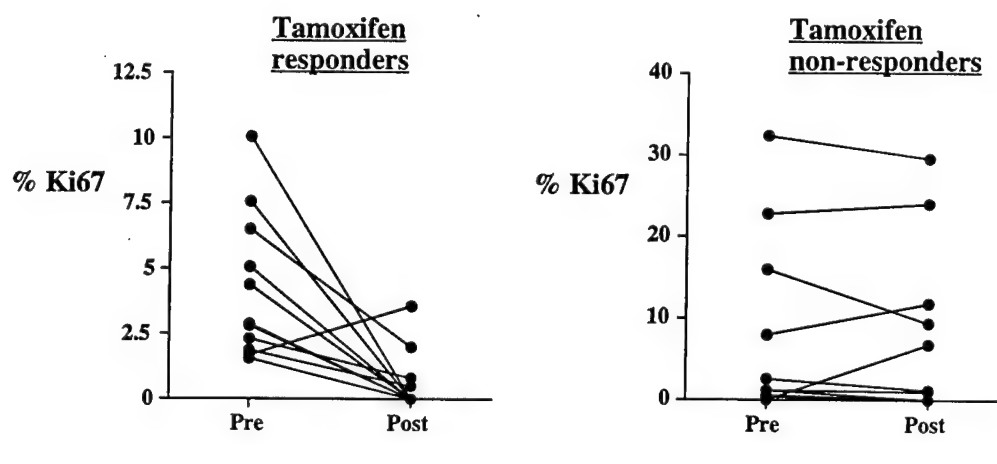


Fig. 2. Change in Ki67 (%) after 14 days of treatment with tamoxifen in 11 responders and 11 non-responders. Measurements were made on cytopins of FNAs.

our first series we reported [12] no significant change in proliferation as measured by Ki67, although there was a trend (median pre-treatment, 19.2%; 24 hours, 15.8%, $p = 0.16$). However, statistical analysis of a total of 35 patients from the two series revealed a highly significant fall (median pre-treatment, 28.5%; 24 hours, 16.9%, $p = 0.009$). Only nine of the 35 patients showed a >50% fall, which makes this change after 24 hours less likely to be usable as an index of response than the change in apoptosis. It does, nonetheless, indicate that it may be valuable to study potential determinants of change in proliferation within such samples (e.g. cyclins and cyclin-dependent kinases and their inhibitors).

We also found that the c-erbB2 status of the tumour was significantly related to the change in apoptosis [13]. Nine of the 39 patients (23%) were c-erbB2 positive. In this group the mean increase in apoptotic index at 24 hours (as a percentage of baseline) was 34% compared with 245% in the c-erbB2-negative group ($p = 0.02$). These data emphasise that the changes in apoptosis and proliferation may themselves be considered appropriate indices of biological response when assessing markers which may be associated with chemoresistance. This point is emphasised by data from our xenograft experiments [14], which showed that marked decreases in proliferation and/or increases in apoptosis can occur without being associated with what would be described as an objective regression in a clinical context (Fig. 3). Whilst such changes may be expected to slow tumour growth, initial rates of apoptosis and proliferation will need to be included into any algorithms to predict actual regression, perhaps in the form of a growth index [14].

Tumour Volume (mm³)

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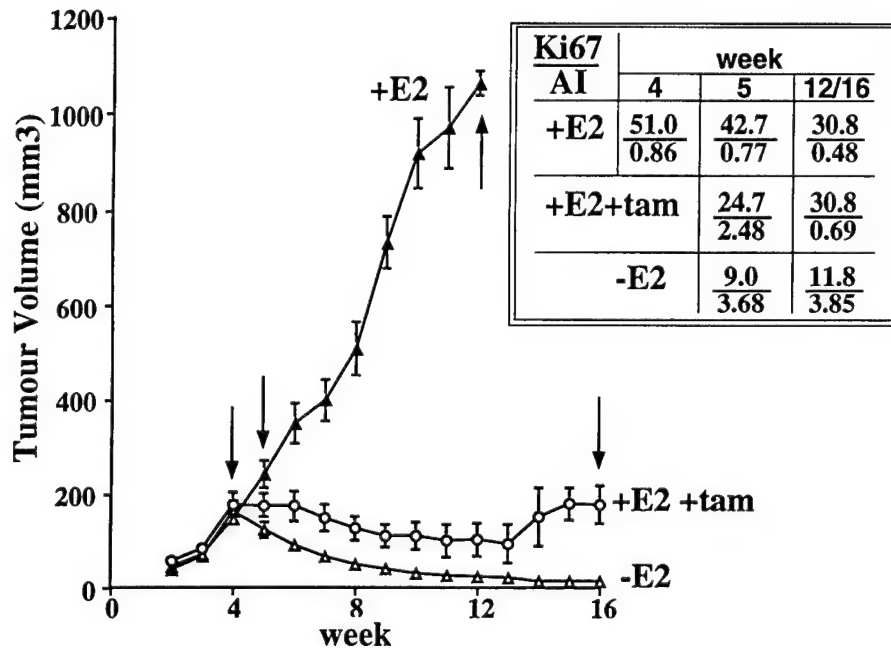


Fig. 3. Growth curves of MCF7 human breast cancer xenografts grown with oestradiol (E2) support for 4 weeks; then treatment was either continued with E2 alone or tamoxifen treatment was added or E2 was withdrawn. Ki67 (%) and AI (%) were measured at the points shown by the arrows and are shown as Ki67/AI ratios. It is notable that there are substantial changes in both Ki67 and AI after one week of tamoxifen and, although growth ceased, there was no objective regression of tumours.

We have extended our studies of changes in Ki67 with chemotherapy by making measurements after 21 days in patients treated with MM (mitoxantrone, methotrexate) [15]. Eleven of the 12 patients who eventually responded to therapy showed a decrease in Ki67: pre-treatment median levels of 35.2% fell to 5.2% after 21 days, with a mean fall of 68% (percentage of baseline). In contrast, all four of the non-responders showed an increase (Fig. 4). Although the numbers of patients in the non-responders group was very small, these data suggest that change in proliferation after this time is a potential intermediate response indicator, as is argued for treatment above.

Apoptotic index, proliferation (Ki67) and bcl-2 expression have also been measured after three months of ECF in comparison with pre-treatment values. It should be noted that the measurements could not be made in nearly half the patients because there was insufficient residual tumour tissue at operation (c. 20% complete clinical response), leaving 20 patients in whom paired analyses could be made [7]. Median Ki67 levels fell from 8.0% (range: 0.3-41.3%) to 1.3% (0.2-21.9%, $p = 0.004$) and apoptotic index from 0.59% (0.21-1.8%) to 0.24% (0.10-0.87%, $p = 0.004$). Thirteen of the 20 patients were positive for bcl-2 pre-

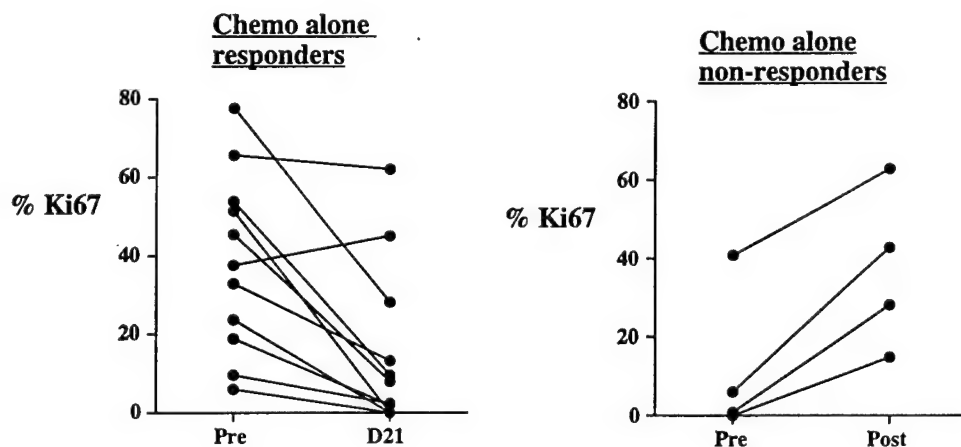


Fig. 4. Change in Ki67 (%) after 21 days of treatment with MM chemotherapy in 12 responders and 4 non-responders. Measurements were made on cytopins of FNAs.

treatment and remained so after treatment; five of the seven patients who were negative pre-treatment were positive after three months' treatment. Overall, the median pre-treatment score of 56% cells positive increased to 80% ($p = 0.03$).

The change in proliferation and apoptosis is particularly well illustrated by comparison of the scattergrams showing the correlation between apoptotic index and Ki67 pre-treatment and after three months of ECF (Fig. 5) [16]. Prior to

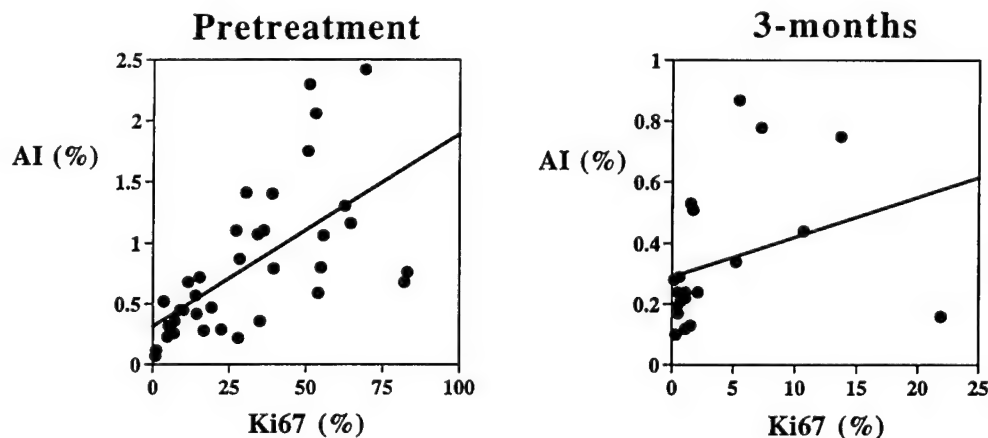


Fig. 5. Relationship between Ki67 and apoptotic index in human breast carcinomas before and three months after the start of chemotherapy. In both cases the relationships were statistically significant: pre-treatment, $\rho = 0.723$, $p < 0.0001$; 3 months, $\rho = 0.492$, $p = 0.03$. The regression lines are only indicative as the data are not normally distributed.

the therapy there is a strong relationship between the two parameters ($\rho = 0.723$, $p < 0.0001$), which has been reported by others [17]. After treatment, a weaker but still statistically significant relationship persisted ($\rho = 0.492$, $p = 0.03$), with the majority of the patients having a very low apoptotic and proliferative activity. Thus for those patients who have sufficient tissue for analysis the residual cell population appears to be quiescent or dormant: there is little proliferation but there is also little cell death. The increase in bcl-2 expression is consistent with this protein's anti-apoptotic function, but cannot be necessarily considered as causative of the reduced apoptosis. Further study of the mechanisms which may underlie the phenotype of this residual population should be a priority.

Chemoendocrine therapy

Biological relationships between molecular markers and therapy in breast cancer may be best studied when the treatment is simple (e.g. single agent). However, if the objective of therapy is to achieve maximal downstaging, with the ultimate aim being complete pathological response, then combination therapy is inevitable at present. Recent overview analyses [18,19] have emphasised the additive benefit from combination chemotherapy and tamoxifen. We have therefore conducted clinical studies of chemoendocrine therapy and derived a substantial amount of biological data from one study in particular, in which 300 patients were randomised to either adjuvant or three months neoadjuvant MM plus tamoxifen (MMT). The clinical data are reported in detail elsewhere in this volume [see chapter by Assersohn & Powles].

The relationship between response to MMT and six immunohistochemical markers (ER, PgR, p53, bcl-2, c-erbB2 and Ki67) was examined initially in FNAs taken from between 45 and 80 patients: sample availability and suitability varied between analytes [20]. SPF and ploidy were measured in a further 60 to 71 patients by flow cytometry. The only parameter which shared a significant relationship with clinical response was c-erbB2: 8/14 (57%) positive patients showed a response compared with 29/31 (93%) negative patients, $p = 0.007$. This relationship has been confirmed in a recent update of the data in whom 100 patients were analysed [21] and by a study of c-erbB2 in the residual tumour of the neoadjuvant patients at surgery [22]. The fact that this oncogene is the only marker of the six to be statistically related to outcome from chemoendocrine therapy may be due to the following: (i) ER, PgR and bcl-2 are markers of good response to endocrine therapy with there being a trend to poor response to chemotherapy; (ii) reverse relationships, i.e. good response to chemotherapy and poor to endocrine therapy, are seen for proliferation (Ki67); (iii) it has become increasingly clear that immunocytochemistry for p53 is unsatisfactory to establish its biological significance [see Lønning et al. in this volume].

We have assessed the change in Ki67 in FNAs taken from 35 patients before and 21 days after starting treatment with MMT [15,23] in analogous studies to

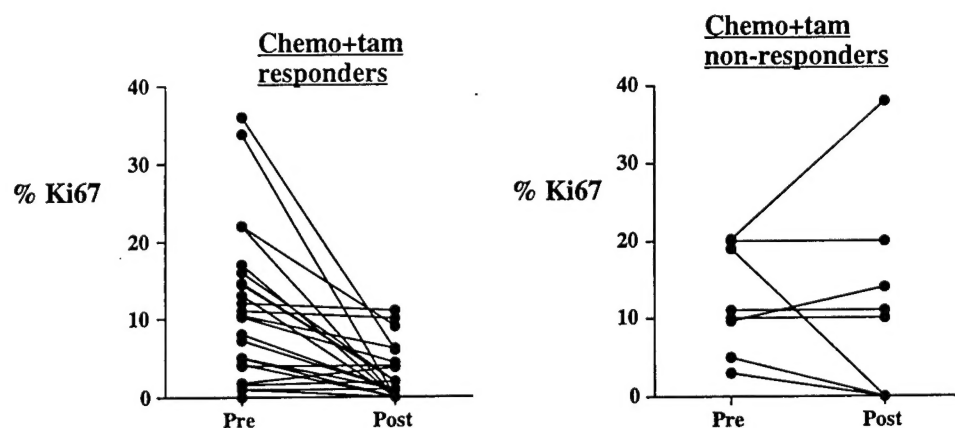


Fig. 6. Change in Ki67 (%) after 21 days of treatment with MMT chemoendocrine therapy in 27 responders and 8 non-responders. Measurements were made on cytopins of FNAs.

those cited above for tamoxifen and MM when applied separately. Again a strong relationship between decrease in proliferation and response was found (Fig. 6). In an earlier smaller study no significant relationship was found between response and change in ER, PgR, p53, bcl-2 or SPF [23].

At the end of three months' chemoendocrine therapy, as with chemotherapy (see above) both Ki67 and apoptotic index were lower than seen in a randomised control group of untreated tumours. In the treated tumours, both Ki67 and AI showed a significant relationship with response (Table 2) [24], with patients who had shown an objective response having significantly lower values than those who had no change or progressive disease. These results are clearly consistent with the observations made above that substantial falls in proliferation occur in responders but not in non-responders to chemoendocrine therapy.

Table 2. Median levels of Ki67 and apoptotic index (AI) in breast carcinomas after three months' treatment with chemoendocrine therapy (MMT) and their relationship with response.

	CR/MRD	PR	NC/PD	P
Ki67 (%)	1.25	2.9	19.6	0.016
n	32	25	12	
AI (%)	0.15	0.28	0.48	0.008
n	23	20	8	

CR, complete response; MRD, minimal residual disease; NC, no change; PD, progressive disease; n = number of patients. Not all samples had sufficient cells for both analyses.

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A further finding obtained by comparing samples from patients receiving no presurgical therapy and those receiving three months of MMT was that angiogenesis levels were lower in the pre-treatment group. The median Chalkey score in 90 MMT patients was 5.7 (95% CI 5.3 - 6.0) and in 105 untreated patients 6.3 (95% CI 6.0 - 6.7; $p = 0.025$). It is not known whether these differences in microvessel density are due to therapy directly causing a reduction in angiogenesis or are secondary to tumour regression [25].

Discussion

Data from the NSABP-B18 study [see review in this volume by Assersohn & Powles] has demonstrated that clinical response to primary chemotherapy is a good indicator of long-term outcome, with pathological complete response being the strongest determinant. Thus, biomarkers that are measured prior to treatment or changes in biomarkers shortly after starting treatment that are strongly predictive of response may themselves be markers of long-term outcome, although this would need to be demonstrated directly.

Our studies have identified a small number of parameters which merit further study in this context. Increases in apoptosis and decreases in proliferation are common factors in response to endocrine therapy and chemotherapy. Relationships with response have yet to be confirmed for apoptosis but are consistent across studies for changes in Ki67 after two to three weeks. The predictive power of these changes needs to be established in larger studies. c-erbB2 status is indicative of a poor response to both chemo and endocrine therapy. Further studies in relation to anthracyclines and taxanes may make this a clinically utilisable observation.

The early changes in proliferation and apoptosis which occur within a day of starting chemotherapy present this as a very valuable scenario for the investigation of the molecular determinants of these changes (e.g. p53, p21^{cip1}, bcl-2 family, activation of caspases, cyclins); indeed, certain of these parameters may be revealed as earlier and/or more sensitive markers than proliferation and apoptosis themselves. The application of microarray analyses to clinical samples taken during this period at the time of maximal clinical response may reveal yet more clinically important biological data. Overall it seems clear that molecular and biochemical analyses in association with controlled clinical trials of primary medical therapy will remain one of the most valuable areas of translational research in breast cancer.

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